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Pre- and post-embedding immunogold labeling and electron microscopy in plant host tissues of three antigenically unrelated MLOs: primula yellows, tomato big bud and bermudagrass white leaf

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Abstract

Methods are described for pre- and post-embedding immunogold labeling of mycoplasmalike organisms (MLOs) in thin sections of infected plants. Antisera against primula yellows (PY), tomato big bud (TBB) and bermudagrass white leaf (BGWL) MLOs, and a monoclonal antibody (mab) against PY were tested with the three serologically unrelated MLOs. Labeling was specific for each MLO and was localized to the outer surface of the MLOs. The antisera performed well in both pre- and post-embedding experiments; the mab reacted well in pre-embedding conditions but gave no labeling with post-embedding. Glutaraldehyde fixation reduced levels of labeling in post-embedding conditions. The results show that these techniques can be used to differentiate MLOs reliably, and extend the usefulness of electron microscopy in this area.

Introduction

A classical approach to identifying diseases caused by mycoplasmalike organisms (MLOs) in plants is thin sectioning and electron microscopy of the phloem tissues, within which the MLOs are normally easily distinguished. However, as one MLO looks much like another, different types cannot be differentiated [Hiruki, 1988; Maramorosch and Raychaudhuri, 1988; Whitcomb and Tully, 1989; Clark, 1992]. Moreover, when only a few membrane-bound 'vesicles' are detected in diseased phloem cells which may themselves be in poor condition, it can be difficult to say whether such vesicles represent MLOs or not.

Immunogold labeling of thin sections (post-embedding labeling) and labeling of bulk tissues before embedding and sectioning (pre-embedding labeling) both have potential to resolve this problem, and in recent years a number of antisera have been raised against MLOs [see reviews by Clark, 1992; Hiruki, 1988; Kirkpatrick, 1989; Markham, 1988; Sinha, 1988; and the papers of Saeed et al., 1993; Sarindu and Clark, 1993]. Monoclonal antibodies (mabs) have also been prepared [Lin and Chen, 1986; Chen and Jiang, 1988; Clark et al., 1989; Garnier et al., 1990; Guo et al., 1991; Jiang et al., 1989; Schwartz et al., 1989; Shen and Lin, 1993; Chen et al., 1993].

There have however been no reports on pre-embedding labeling of MLOs, and only one full paper on post-embedding, which concerned an MLO in its insect vector [Lherminier et al., 1990]. There are two other preliminary reports of aspects of the present work [Lherminier et al., 1992; Milne, 1992], and Benhamou [cited in Benhamou, 1989] has described gold labeling of MLOs in sections using lectins. Methods for immunotrapping and gold labeling of MLOs in crude sap

extracts have been detailed by Vera and Milne [1994].

We here present methods for pre- and post-embedding immunogold labeling of MLOs in plant tissues, and the results obtained with three serologically unrelated MLOs.

Materials and methods

MLO isolates. Primula yellows (PY) and tomato big bud (TBB) came from Horticultural Research International, East Malling, UK but PY was originally from the John Innes Institute, Norwich, UK and TBB from the Plant Research Institute, Burnley, Australia [Clark et al., 1989]. We also tested an MLO causing a TBB-like disease in tomatoes in Sardinia [Lovisolo et al., 1982]. Bermudagrass white leaf (BGWL) MLO [Bar-Joseph et al., 1975; Maramorosch et al., 1975; Sarindu and Clark, 1993] was from East Malling.

PY and TBB were propagated in periwinkle (*Catharanthus roseus*). The Sardinian MLO was propagated in tomato cv. Marmande. BGWL was maintained by vegetative propagation of infected bermudagrass (*Cynodon dactylon*), and healthy bermudagrass was grown similarly. MLO-infected tissue samples were taken from young shoots showing strong symptoms, on plants infected by grafting about one month earlier.

Polyclonal and monoclonal antibodies. The PY mab, PY antiserum and two TBB antisera were described by Clark et al. [1989]. TBB antiserum #1 was prepared using infected tomato, whereas #2 was prepared using TBB from *C. roseus*. The BGWL antiserum was that of Sarindu and Clark [1993]. Antisera were absorbed with healthy plant preparations [Clark et al., 1983]. Antisera against healthy periwinkle [Clark et al., 1983] and healthy bermudagrass [Sarindu & Clark, 1993] were used as controls. Three plant virus antisera from the Torino collection, also used as controls, were chosen because they all originally had high titres against healthy antigens but had been cross-absorbed like the MLO antisera. IgG was prepared from all antisera, lyophilized, and stored at –20 °C or diluted to 0.5–2.0 mg/ml in phosphate-buffered saline containing 1/5000 sodium azide (w/v) and stored at 4 °C. For labeling experiments, the IgGs were diluted to 20–200 (routinely 50) µg/ml.

Labeling was done using 5 nm gold conjugated either to goat antirabbit (GAR-G5) or goat anti-mouse (GAM-G5) IgGs (Auroprobe One, Janssen Biotech N.V., now amersham International, UK).

Post-embedding procedures

These were based partly on the work of Berryman and Rodewald [1990], Lherminier et al. [1990] and Viale et al. [1985].

Fixation. Pieces of leaf were immersed at room temperature (RT) in 4% (w/v) formaldehyde (FA), 0.1% (w/v) glutaraldehyde (GA) and 0.5 mM calcium chloride in 0.1 M potassium phosphate buffer, pH 7 (PB). The fixative was vacuum-infiltrated, and after 1 h edge tissue was removed and small leaf veins isolated; these were treated with fresh fixative on ice in a shaker for a further 1 h, washed 4 × 10 min in PB plus calcium chloride, containing 3.5% (w/v) sucrose, and shaken for a further 1 h in the same solution plus 50 mM ammonium chloride, which served to quench any remaining free aldehyde groups. Tissues were now washed 4 × 15 min in cold 0.1 M maleate buffer pH 6.8 (MB) containing sucrose as above, and after a further change, were left in this mixture overnight. Finally the tissue was soaked for 2 h in MB-sucrose containing 2% (w/v) uranyl acetate (UA), final pH 6.4; (maleate was used since phosphate is incompatible with uranyl acetate).

In some experiments, all steps proceeded in MB in place of PB, so that washing to remove PB could be avoided. At the same time the level of calcium chloride was raised to 1 mM.

Experiments were done with PY to assess the effects of a break in the protocol (simulating transport of field samples) after fixation. Tissues were either held in fixative for 48 h in the cold, or rinsed in PB and held in this for 48 h in the cold. At the end of this time, similar tissues were freshly fixed, and all three treatments were then processed together.

We also varied the standard protocol by testing levels of 0, 0.1, 1 and 2.5% GA.

Dehydration and embedding. Fixed tissue was transferred to 50/50 (v/v) acetone/water at 0 °C,

after which all steps proceeded at –20 °C, the tissues being tumbled on a rotor in a freezer. Tissues were brought to 90% acetone, then infiltrated with LR Gold resin (LRG; London Resin Co. Ltd., Woking, UK). After passage in LRG plus 0.5% (w/v) Lowicryl initiator (Biorad), the tissues were embedded in closed BEEM capsules and polymerized under indirect UV light.

Incubation and staining of sections. A hole 2 mm in diameter was punched in a small wedge of thin plastic sheet, and this tool (the 'goldfish') was held by the 'tail' while the 'eye' was lowered over a group of sections floating on the waterbath. A lens of liquid bearing the sections was then transferred from one solution to another. Finally, a Formvar-filmed copper grid was lowered onto the sections.

Processing (all steps at RT except incubation with the primary antibody) was as follows: blocking for 15 min in NGS-TBS-BSA (normal goat serum diluted 1/30 (v/v) in 0.05 M Tris-HCl, 0.15 M NaCl, containing 0.2% (w/v) BSA, pH 7.6); incubation overnight at 4 °C with primary antibody (50–200 µg/ml) diluted in NGS-TBS-BSA; rinsing (5 × 3 min) on NGS-TBS-BSA; incubation for 60 min on GAR-G5 or GAM-G5, diluted 1/50 in TBS pH 8.2 containing 1% BSA; rinsing (5 × 3 min) on TBS pH 8.2; rinsing (5 × 1 min) on deionized water; staining for 60 min with 5% (w/v) aqueous uranyl acetate; rinsing (5 × 3 min) on deionized water; collection of the sections on filmed grids, and observation.

Instead of overnight incubation at 4 °C with primary antibody, a 3 h incubation at RT was tried. The TBS-BSA routinely used to dilute the GAR or GAM was normally at pH 8.2, but in some experiments the pH was 7.4, or 8.5.

Controls. Each MLO-infected tissue was tested with its homologous antibody and with the antibodies to the other two MLOs. TBB-infected tissue was in addition tested with the antiserum to healthy *C. roseus* antigens, and BGWL-infected tissue with the antiserum to healthy bermudagrass antigens. TBB-infected tissues were tested with the three antisera against plant viruses. The MLO antibodies were tested with healthy *C. roseus* and *C. dactylon* tissues.

Since MLOs are strictly confined to the phloem, it was easy to find uninfected nonphloem cells adjacent to those containing MLOs, and these also served as controls.

Pre-embedding procedures

These were based partly on the work of Morgan et al. [1961] and Shalla and Amici [1967].

Tissue preparation. Young *C. roseus* leaves carrying either PY or TBB were chilled on ice; veins were isolated with a razorblade and sliced transversely into discs 0.2–0.4 mm thick.

Antibodies. The IgGs tested were the anti-PY mab and from TBB antiserum #1, and were used at 10 µg/ml.

Labeling. A group of discs was immersed in 50–200 µl of primary antibody diluted in PB, in a well of a microtitre plate agitated at 4 °C for 60 min. After a rinse (4 × 3 min) in cold PB, discs were incubated as before in 200 µl of GAR-G5 or GAM-G5 diluted 1/50 in PB, for 60 min. The discs were then rinsed (4 × 3 min) in cold PB.

Fixation and embedding. The discs were fixed in the cold on a shaker as above, in 2.5% (w/v) GA in PB for 60 min, then in 0.1% (w/v) osmium tetroxide in PB for 60 min. The discs were embedded in Epon [Milne, 1970], after orientation so they could be sectioned longitudinally with respect to the vein axis.

Sectioning and staining. Longitudinal thin sections that included the phloem were cut so that both ends of the disc exposed to antibody were included in one section; they were stained in lead citrate (uranyl acetate staining being already done before embedding).

Controls. PY- and TBB-infected periwinkle were tested in heterologous combinations with the PY mab and with the anti-TBB IgG. The level of labeling over host tissues and especially host membranes was carefully observed.

Electron microscopy. All preparations were examined at 60 kV in a Philips CM10 electron microscope.

Results

Post-embedding

Assessment of the method. The method used gave relatively clean and consistent results. Nonspecific background labeling was low (Figs. 1 and 2), with a few exceptions (Fig. 2B), especially after adoption of the goldfish system, which was no more laborious than processing grids with sections already mounted on them.

Use of 1% or 2.5% of GA instead of 0.1%, for fixation, abolished all labeling of the MLOs; on the other hand, omitting GA entirely led to very poor structural preservation.

The antibodies to PY, TBB and BGWL labeled only their respective MLOs. Cells not containing MLOs were not labeled except that there was a tendency for cell walls and chloroplasts to be labeled if antisera were not rigorously cross-absorbed with healthy material. With one antiserum (see below) nucleoli were clearly labeled.

Use of MB instead of PB during the first half of the fixation (and doubling the level of Ca^{++}) appeared not to influence the results; however, use of MB throughout simplified and shortened the protocol.

The effects of a 48 h break in the protocol after fixation were as follows. Material held in cold PB during the interval did not appear to suffer either antigenic or structural damage, compared with material whose processing was uninterrupted. However, material held in the FA-GA fixative for 48 h suffered antigen damage, as label intensity fell to about one quarter.

Incubation with primary antibody for 3 h at RT gave slightly less labeling than incubation at 4 °C overnight (15 h). Dilution of the GAR-G5 in Tris

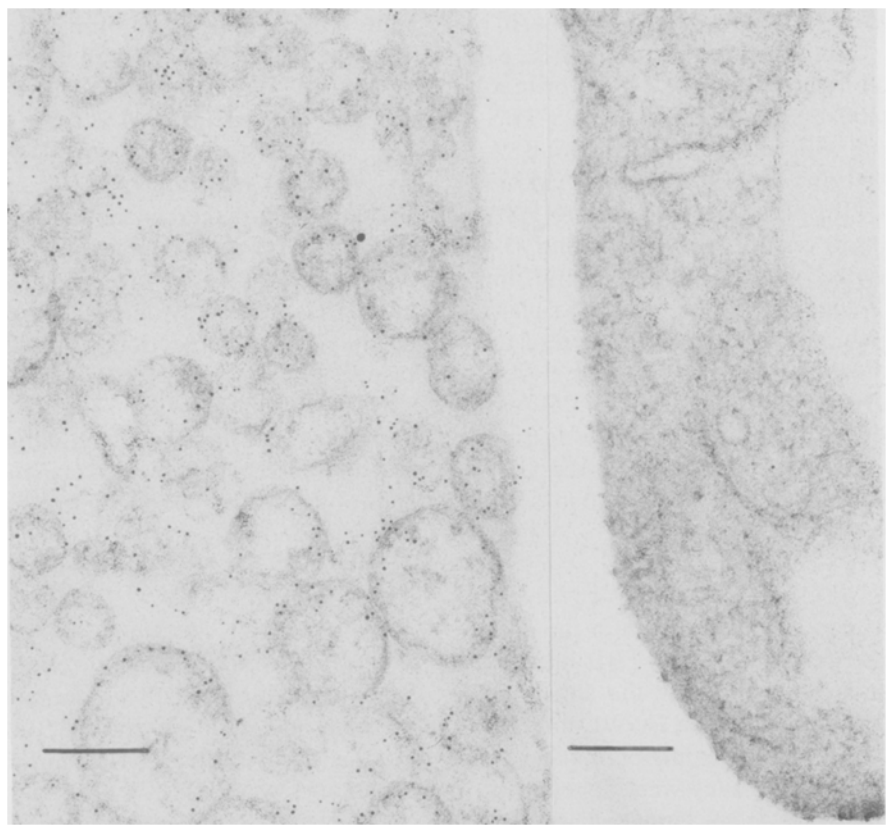

Fig. 1. Post-embedding preparation of tissue containing PY MLOs, treated with homologous polyclonal IgG at 50 µg/ml. The Fig. shows two adjacent cells, with part of the common cell wall eliminated from the photograph. The left hand cell contains labeled MLOs; the right hand (nonphloem) cell is unlabeled and presumably uninfected. Bars = 200 nm.

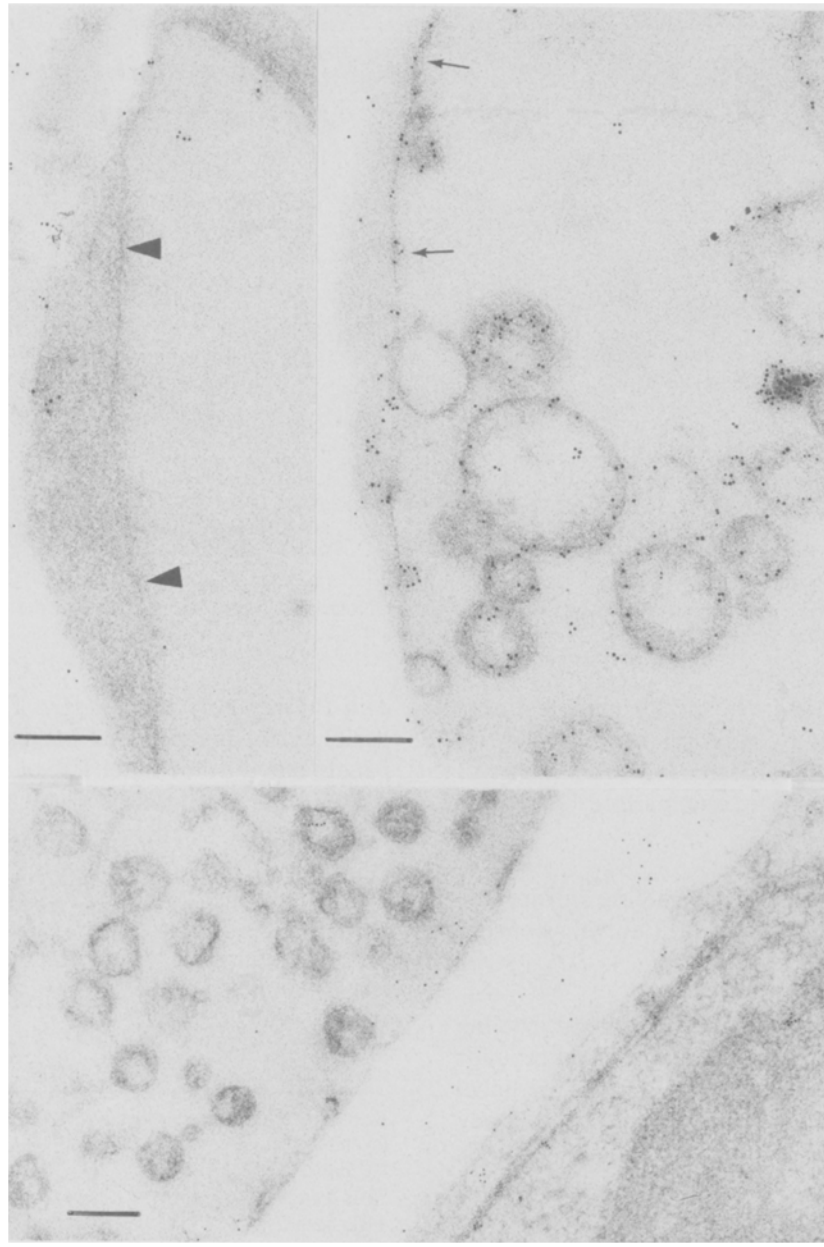

Fig. 2. Post-embedding preparations. (A) BGWL MLO-containing section treated with homologous IgG at 150 μg/ml; parts of two adjacent cells photographed on the same negative. The MLOs (right panel) are labeled. In the MLO-containing cell, the plasmalemma also appears to be labeled (small arrows) whereas in the presumed uninfected cell (left panel) the plasmalemma was not labeled (arrow heads). (B) Parts of two cells in a PY-infected plant, treated with anti-BGWL IgG at 150 μg/ml. Note absence of label on the MLOs, some label on the cell wall, and very little label on the nucleus and nucleolus. Bars = 200 nm.

at pH 7.4 or 8.5 instead of 8.2 made no detectable difference.

Levels of structural preservation of the MLOs obtained varied considerably, from poor (Fig. 2B) to relatively good (Figs. 1 and 3). This may partly reflect the variable condition of the MLOs in different host cells. However, structural preservation of the host tissues was also variable. MLOs that

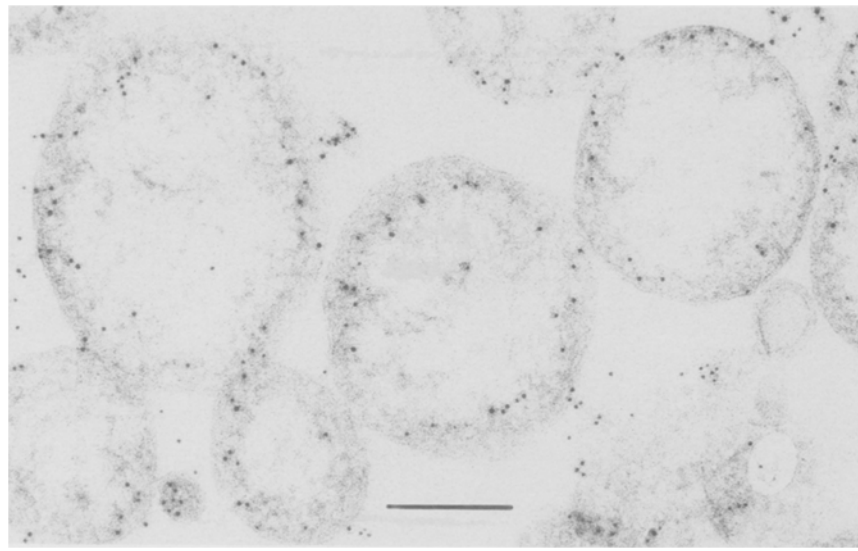

Fig. 3. TBB MLOs treated with homologous IgG (#1) at 50 µg/ml. Note label where the periphery of the MLOs is exposed at the surface of the section. Bar = 200 nm.

were poorly preserved structurally were nevertheless labeled clearly by appropriate antibodies.

Results with the different combinations of antigen and antibody are summarised below and in Table 1.

Anti-PY mab. This gave no labeling in repeated tests with the homologous MLO, using concentrations of IgG up to 150 µg/ml.

Anti-PY polyclonal IgG. This gave good labeling of homologous MLOs and low backgrounds over host cell components (Fig. 1); the label was confined to the MLO surface. The antibody occasionally labeled the plasmalemma of cells carrying the MLO, though in apparently uninfected adjacent cells the membrane was not labeled. This may indicate that some of the MLO antigen became mobilized and migrated to the plasmalemma.

With BGWL-infected material, there was essentially no label either on the MLOs or over host tissue. With TBB-infected material, label over host tissues were again almost absent but a low level of label was found over the body of the MLOs. Such label was absent over the MLO periphery. With the Sardinian MLO, this antibody gave no reaction.

Anti-BGWL polyclonal IgG. This labeled the periphery of homologous MLOs (Fig. 2A, right panel) but did not label PY or TBB (Fig. 2B). As with PY and its homologous polyclonal, the plasmalemma of those cells carrying BGWL

Table 1. Labeling of bounding membranes of MLOs by different IgG preparations

IgG against	MLOs			
	PY	TBB	BGWL	Sard. tomato
Post-embedding experiments				
PY mab	–	–	–	ND
PY poly	+	?	–	–
TBB #1 poly	–	+	–	–
TBB #2 poly	–	+	–	–
BGWL poly	–	–	+	ND
Healthy *C. roseus*	+	+	ND	ND
Healthy *C. dactylon*	ND	ND	+	ND
Carnation vein mottle virus	ND	–	ND	ND
Poplar mosaic virus	ND	–	ND	ND
Bean yellow mosaic virus	ND	–	ND	ND
Pre-embedding experiments				
PY mab	+	–	ND	ND
TBB #1 poly	–	+	ND	ND

+ = positive labeling; ? = apparently non-specific label over interior of MLO; – = no labeling; ND = not done.

MLOs were sometimes labeled (Fig. 2A, right panel) though in apparently uninfected adjacent cells they were not (left panel).

Anti-TBB polyclonal IgG #1. This labeled the periphery of homologous MLOs but not the internal parts (Fig. 3); label over host tissues was essentially absent. There was no labeling of PY, BGWL or the Sardinian MLO.

Anti-TBB polyclonal IgG #2. This labeled the periphery of homologous MLOs but did not label PY, BGWL or the Sardinian MLO. There was some labeling of cell walls, and clear labeling of nucleoli of both healthy and infected tissues.

IgG directed against healthy periwinkle. We expected to see host tissues heavily labeled but the MLOs unlabeled. In fact we observed, with PY- and TBB-infected tissues, large amounts of label over host tissues, but considerable label also on the MLOs.

IgG directed against healthy bermudagrass. BGWL-infected tissues gave results similar to the above.

IgGs against three plant viruses. These gave, with TBB-infected material, clean results with no label on the MLOs and a very low background.

Pre-embedding

Assessment of the method. Host tissues did not survive well after being sliced and incubated for some hours in the cold without fixation. However, the MLOs proved resistant both morphologically and antigenically. They were often retained within the opened phloem cells (Fig. 4), but were sometimes lost. Where MLOs were retained, labeling was intense and specific (Figs. 5 and 6). The results are summarised below and in Table 1.

Anti-PY mab. In contrast to the negative result obtained with post-embedding, this antibody, in the pre-embedding system, strongly labeled the surface of homologous MLOs, with no labeling of host elements (Fig. 5). There was no labeling of TBB MLOs.

Anti-TBB polyclonal IgG #1. This strongly labeled the surface of TBB MLOs (Fig. 6A) but did not label PY MLOs (Fig. 6B). Host tissues were essentially unlabeled (Figs. 6A and B).

Discussion

Our results show that both pre- and post-embedding immunogold labeling methods can be used to distinguish between serologically differing MLOs

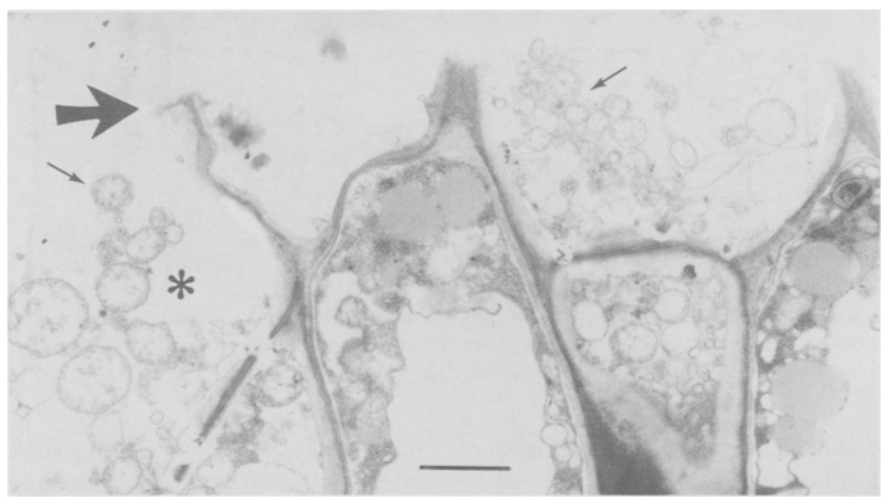

Fig. 4. Pre-embedding preparation showing phloem infected with PY MLOs, sliced open and exposed to PY monoclonal antibodies. The plane of the slice is indicated by the large arrow. Exposed MLOs are indicated by small arrows. The area near the asterisk is enlarged in Fig. 5. Bar = 1 μm.

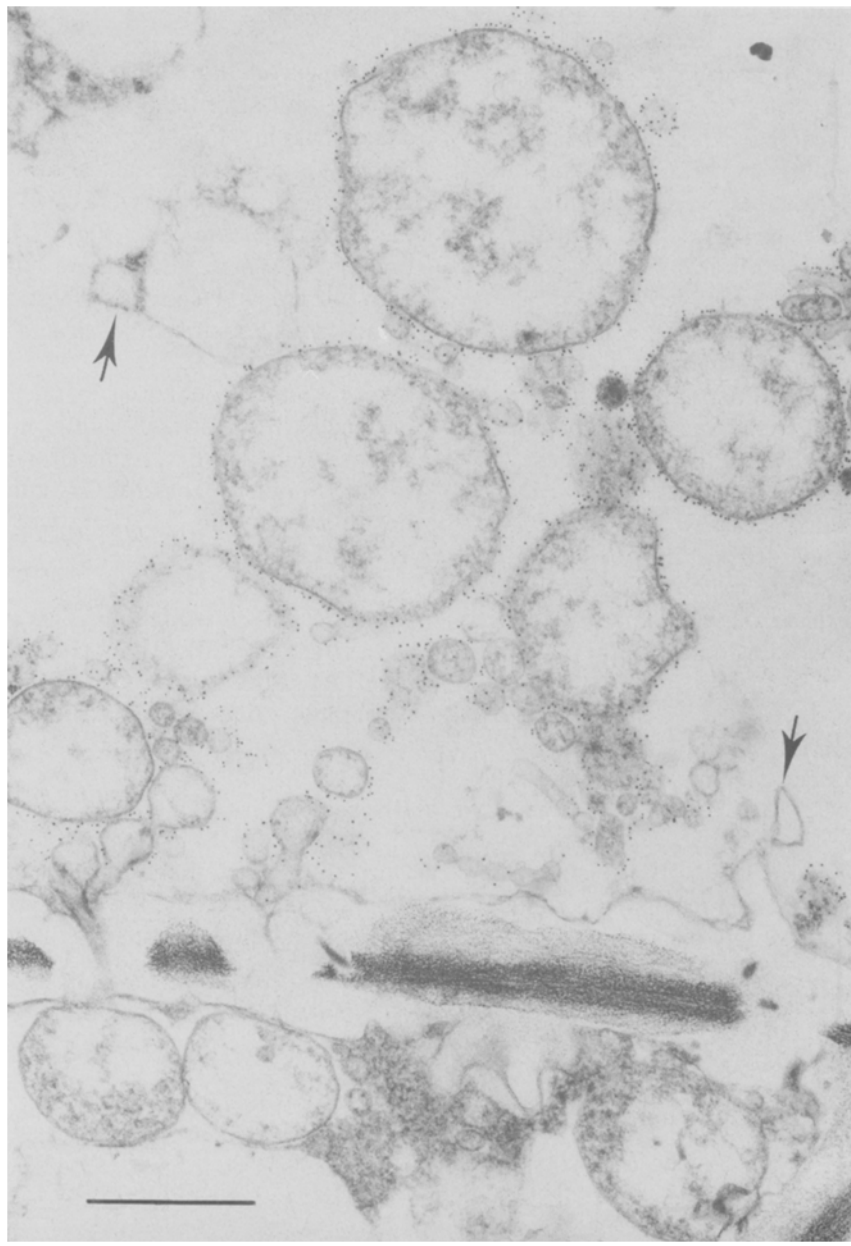

Fig. 5. Part of Fig. 4 enlarged. The MLOs external to (above) the sieve plate are labeled whereas host membranes (arrows) are unlabeled. Material below the sieve plate was not exposed to antibody and is unlabeled. Note the relatively good preservation of the MLOs and their excellent reactivity with this monoclonal. Bar = 500 nm.

in plant tissues. With both methods, some background labeling of host elements can be tolerated (i.e. ignored) since the MLOs are recognized by their morphology. Mitochondria, most easily confused with MLOs because of their size and shape, especially if the cristae are indistinct, were never labeled. Thus labeling should be a useful means of verifying the presence of MLOs in doubtful cases.

We confirm the result of Lherminier et al. [1990] that the best mixture of aldehydes to use for preservation of MLO structure and antigenicity

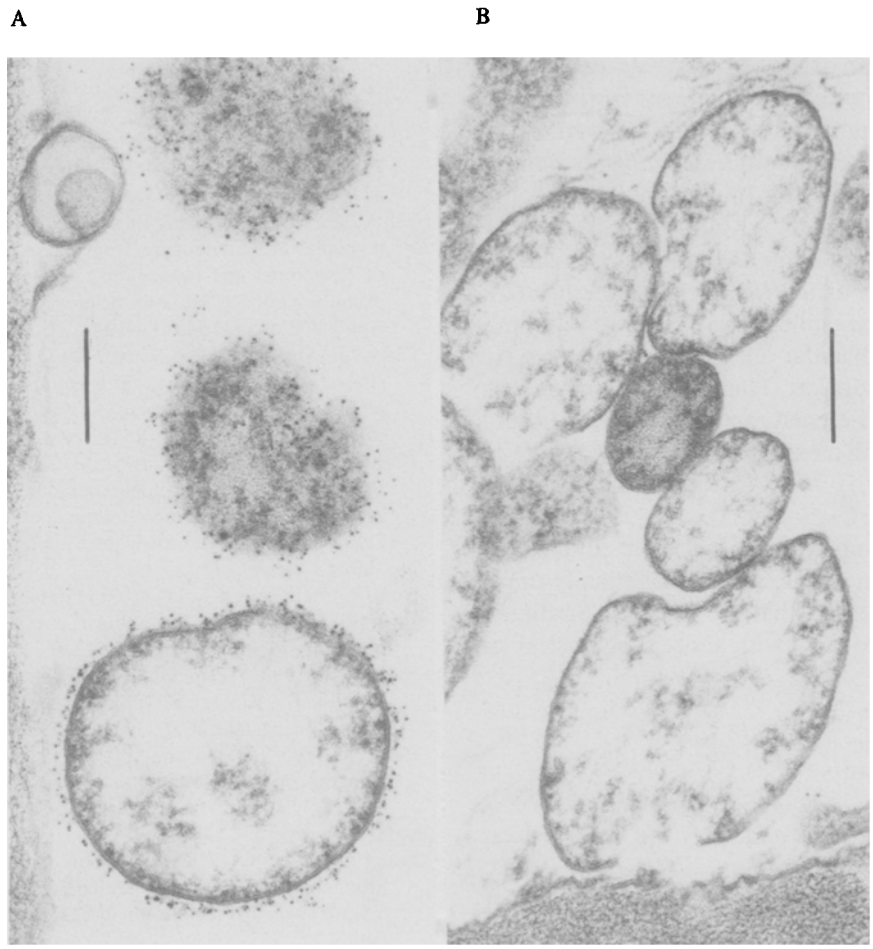

Fig. 6. Pre-embedding preparations. (A) Part of a cell containing TBB MLOs exposed to homologous IgG #1. Note three strongly labeled MLO profiles; the cell wall and cell membrane are unlabeled. (B) PY-infected cell exposed to anti-TBB IgG #1. Note the almost total absence of label on MLOs, host membranes and *p* protein. Bars = 200 nm.

in post-embedding systems was 4% formaldehyde plus 0.1% glutaraldehyde.

With the PY polyclonal on PY-infected cells and with BGWL IgG on BGWL-infected cells, there was occasional labeling of the plasmalemma as well as of MLO membranes; we suggest this occurred because of migration of imperfectly immobilized MLO antigens; these IgGs did not label the plasmalemma of cells not containing MLOs. With the polyclonals against healthy periwinkle and healthy bermudagrass, there was some labeling of MLO membranes in addition to the expected labeling of host tissues, and this result is also most simply interpreted as migration of host antigens to the MLO surface. Although the general phenomenon of antigen migration is important and well known [Hayat, 1989], it did not affect specific recognition of MLOs in cells; it might however cause difficulty in distinguishing two antigenically unrelated MLOs in mixed infections in the same cell, if the antigen of one MLO should migrate to the surface of the other.

Post-embedding lends itself more easily than pre-embedding to the probing of a given MLO in different ways, since a few sections from the same block can form the basis of a series of experiments. With pre-embedding, setting up parallel experiments is more laborious and more costly in use of antibody; in addition, loss of MLOs from some opened cells may be a problem.

Advantages of pre-embedding are that labeling occurs in three dimensions and in our experience

was more intense than with post-embedding; also the ultrastructure of the MLOs was better preserved. Labeling can be done on unfixed and perhaps unaltered antigens; this allowed us to show that the PY mab could recognise its homologous antigen, although in our post-embedding system it did not.

With pre-embedding, only antigens exposed on the outer surface of the MLO can be recognized; the fact that pre-embedding labeling was successful shows that the antigens in question are indeed on the surface. This confirms what was seen, but not so clearly, in the post-embedding experiments; it also explains why it is possible to detect and identify MLOs using immunocapture PCR [Rajan and Clark, 1994].

Our findings support the reported result that PY, TBB and BGWL antigens are serologically unrelated. It is of interest that the MLO in Sardinian tomatoes failed to react with antisera of Australian TBB.

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